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Istituto Ortopedico Rizzoli di Bologna
Istituto di Ricovero e Cura a Carattere Scientifico

"Tumor microenvironment in patients with localized osteosarcoma treated with mifamurtide: a translational study"

Study code	**Micr-OS**
Sponsor's Name and Address:	Istituto Ortopedico Rizzoli Via di Barbiano 1/10 40136 Bologna Italy
Study Number/Version/Date:	Vers 1.0 27 March 2018
Coordinating Center:	IRCCS Istituto Ortopedico Rizzoli Chemotherapy Unit Via Pupilli 1 40136 Bologna, Italy
Coordinating Investigator and address:	Emanuela Palmerini MD Phone :051-6366.199 Email :emanuela.palmerini@ior.it
Transalational Laboratory:	Sant'Orsola Hospital, Bologna/ Department of Experimental, Diagnostic and Specialty Medicine (DIMES) University of Bologna Dr Michelangelo Fiorentino Dr Claudio Agostinelli
Methodology:	Translational study
Type:	Academic
Founding:	None

TABLE of CONTENTS

BACKGROUND

The osteosarcoma bone microenvironment is heterogeneous and consists of osteoclasts, osteoblasts and hematopoietic cells from which monocytes/macrophages derive. All of these cells release multiple growth factors and cytokines with contrasting effects that are not well documented in the context of OS. However, it is widely thought that this microenvironment plays an important role in tumor development.

Indeed, intratumoral accumulation of CD8 T-cells (tumor infiltrating lymphocytes) in pretreatment biopsies was an independent prognostic factor for prolonged survival in patients with localized osteosarcoma treated within ISG-OS1 trial [1]. As for most other tumors, tumor infiltration by antigen presenting cells (APCs) including CD1a DCs and CD68 macrophages has been correlated with poorer prognosis, and tumor PDL-1 expression has been associated with a poorer 5-y event-free survival (EFS) [1,2]. However, other studies have also associated tumor-associated macrophages (TAMs) with reduced metastasis and improved survival in high-grade OS [3-5]. Reasons of this discrepancy might be the macrophage polarization in situ: T helper 1 responses and M1 polarization (pSTAT1) and for T helper 2 responses and M2 polarization (CMAF) [5].

Also, it was demonstrated in soft tissue sarcoma that TAMs express IDO1, which might contribute to their immunosuppressive phenotype [6]. Last, CD4+ regulatory T (Treg) cells expressing CD25 and the transcription factor forkhead box P3 (FOXP3) play indispensable roles for immunological self-tolerance and homeostasis and CD15s (sialyl Lewis x) is specifically expressed by activated, terminally differentiated, and most suppressive FOXP3high Treg cells [7].

Mifamurtide (Mepact®), is approved in the European Union for the adjuvant treatment of localized osteosarcoma based on the result of a phase III trial, demonstrating an improvement of 9% in 3-year EFS when combined with ifosfamide in an unselected population with localized osteosarcoma younger than 30 years (3-year EFS standard arm 71%; 3-year EFS with the addition of both ifosfamide and MTP resulted in a 3-year EFS rate of 78%) [8].

Mifamurtide increases innate immune response and activate macrophage anti-tumoral phagocytosis [8].

No predictive factor for response to mepact were identified.

OBJECTIVE OF THE STUDY

- Characterize the immunephenotype in patients with localized osteosarcoma treated with mifamurtide for primary osteosarcoma

- Correlate the rate of immune cells (TAM and TIL), and the level of the PDL-1 checkpoint with event-free survival (EFS) and overall-survival (OS)

STUDY DESIGN

This is an International, multicenter retrospecitve biological study that will analyze the tumor micro environment in biological archival samples of patients, who were treated with mifamurtide according the protocols for primary localized osteosarcoma (ISG-OS2 and GEIS-33)

POPULATION

Inclusion criteria

1) Patients with primary localized osteosarcoma who received mifamurtide according to ISG-OS2/GEIS-33 trials,
2) Paraffin-embedded tissue tumor (FFPE) blocks from archive available to perform the biological analysis
3) Written informed consent prior to any study-specific analysis and/or data collection

According to the Italian "*Autorizzazione generale n. 9/2016 al trattamento dei dati personali effettuato per scopi di ricerca scientifica"* of the Privacy Tutor (and the corresponding regulation in the other participating countries) the informed consent is not required to be obtained by the deceased subjects, as long as all the other enrolment criteria are met and the study has been approved by the Ethic Committee (refers to INFORMED CONSENT section)

Exclusion criteria

1) Patients with diagnosis different from osteosarcoma
2) Patient not treated with mifamurtide

The study will include male and female patients without limit of age

MATERIAL AND METHODS

For tissue microarray (TMA) construction, a slide stained with hematoxylin and eosin will be prepared from each formalin-fixed, paraffin-embedded (FFPE) sample of pre-treatment biopsy, and representative tumor regions will be morphologically identified and marked on each slide. Tissue cylinders with a diameter of 1.0 mm will be punched from the marked areas of each block and brought into a recipient paraffin block. Five TMAs were constructed. Each tumor sample will

be represented by a minimum of 1 core to a maximum of 5 cores.

From TMA blocks, 4 micron-sections will be cut. The macrophage polarization will be determined in situ by pSTAT1 and CMAF staining, respectively, for the characterization of M1 and M2 subpopulations. Osteoclastic cells (also known as giant cells) will be evaluated independently as giant multinucleated cells by CD68 staining. The presence of checkpoint markers will be assessed with PD1 and PDL-1 antibodies.

In summary, the histological sections will be stained with haematoxylin eosin and the tumor microenvironment will be characterized by applying antibodies directed against fixation resistant epitopes of

- TAM (M1): CD163/CD68/pSTAT1 (CD163 threshold >50%)
- TAM (M2): CD163/CD68/CMAF (CD163 threshold >50%)
- Osteoclast: CD68
- TIL: CD8/Tia1 (CD8 citotoxic)
- Treg: FOXP3/CD15s
- IDO1 / KYN (Ventana SP126 >5%/SD4F2 >1%)
- PD-L1 (Dako 28-8; thresholds >1%, >5%; > 10%), both on tumor cell (TC) and immune-cells (IC)

Treg/CD8 ratio will be also assessed.

Pathologists will be blinded to clinical information. Only cores with tumoral component will be included in the analysis. In case of heterogeneity among cores of the same patient, the highest score will be considered for the analysis.

STATISTICS

For survival analysis the following factors will be correlated with Overall Suvival (OS) and Event Free Survival (EFS): age (pediatric < 18 years vs adult ≥ 18 years), gender, LDH and phosphatase alkaline (PA) levels at baseline (normal vs high), pathologic response (good: chemotherapy-induced tumor necrosis ≥ 90%; poor: chemotherapy-induced tumor necrosis < 90%, [9]), tumoral miroenvironment components, PD-1 expression on IC, and PD-L1 both on TC and IC.

EFS and OS will be estimated according to the Kaplan and Meier method with their respective 95% confidence intervals (CI) and calculated from the first day of chemotherapy administration to death or last follow-up visit. It is expected to include about 80 patients.

ENROLLMENT PROCEDURE

Patients who participated to the ISG-OS2 and GEIS-33 study and considered eligible will be enrolled in the study, after providing a written informed consent.

Due to the high incidence of mortality of the disease under investigation, it would be possible that some eligible subjects will be deceased

DATA COLLECTION

Clinical data will be retrieved by the ISG/OS-2 and GEIS 32 trials.

A protocol-specific CRF reporting the results of the microenviroment analyses will be provided

A CRF is required and should be completed for each included subject.

ETHICS AND QUALITY ASSURANCE

The clinical trial protocol and its documents will be sent before initiating the study to the competent Authorities and Ethics Committees of each participating country for its approval.

The responsible investigator will ensure that this study is conducted in agreement with either the most updated Declaration of Helsinki and all the international and local laws that apply to clinical trials and to patient protection.

The protocol has been written, and the study will be conducted according to the principles of the ICH Harmonized Tripartite Guideline for Good Clinical Practice

(ref: http://www.emea.eu.int/pdfs/human/ich/013595en.pdf).

INFORMED CONSENT

All patients will be informed, by the investigator, of the aims of the study, the possible risks and benefits that will derive from the study participation.

The Investigator must clearly inform that the patient is free to refuse participation in the study and that can withdraw consent at any time and for any reason.

They will be informed as to the strict confidentiality of their patient data, but that their medical records may be reviewed for trial purposes by authorized individuals other than their treating physician.

The informed consent procedure must conform to the ICH guidelines on Good Clinical Practice. This implies that "the written informed consent form should be signed and personally dated by the patient or by the patient's legally acceptable representative".

The Investigator must also sign the Informed Consent form, and will keep the original at the site and a copy of the original must be handed to the patient.

The competent ethics committee for each Institution participating to the study must validate local informed consent documents before the study can be opened. It will be emphasized that the participation is voluntary and that the patient is allowed to refuse further participation in the study whenever he/she wants. This will not prejudice the patient's subsequent care.

Due to the high incidence of mortality of the disease under investigation, it would be possible that some potential eligible subjects will be deceased.

In order to allow and promote the increase in the knowledge of this rare disease that could be beneficial for other patient that are or will be affected, according to the Italian "*Autorizzazione generale n. 9/2016 al trattamento dei dati personali effettuato per scopi di ricerca scientifica"* of the Privacy Tutor and the corresponding regulation in the other participating country, as well as the EU General Data Protection Regulation 679/2016 (that will be applicable from 25 May 2018), the informed consent is not required to be obtained by the deceased subjects according the aforementioned laws/dispositions.

GENERAL PRINCIPLES FOR HUMAN BIOLOGICAL MATERIAL (HBM) COLLECTION

Human biological material (HBM) collection involves the collection and storage of biological material, residual biological material or derivatives in compliance with ethical and technical requirements.

Biological material (tumor tissue) will be centralized and stored at Istituto Ortopedico Rizzoli Pathology Department.

From here, the biological material will be used and stored according with the sample characteristic and applicable regulation.

The Sant'Orsola Hospital (Dr Michelangelo Fiorentino and Dr Claudio Agostinelli, from Department of Experimental, Diagnostic and Specialty Medicine (DIMES)) will perform the research as stated in the protocol "Material and Methods" section

The following principles apply to storage of HBM:

- The Istituto Ortopedico Rizzoli will have a designated person responsible for collection and will act as a communication point
- The collected HBM should be documented, i.e. the amount remaining and its location. act as a communication point

CONFIDENTIALITY

In order to ensure confidentiality of clinical trial data as disposed the national and European applicable regulation, data will be only accessible for the trial Sponsor and its designees, for monitoring/auditing procedures, the Investigator and collaborators, the Ethics Committee of each corresponding site and the Health Authority.

Investigator and the Institution will allow access to data and source documentation for monitoring, auditing, Ethic Committee revision and inspections of Health Authority, but maintaining at all times subject personal data confidentiality as specified in the "Directive 95/46/EC of the European Parliament and of the Council of 24 October 1995".

The Investigator must guarantee that patient anonymity is kept at all times and their identity must be protected from unauthorized persons and institutions.

All patients included in the study will be identified with a numeric code, so that no identifiable personal data will be collected.

The Investigator must have and conserve a patients' inclusion registry where it figures the personal data of the patient: name, surname, address and corresponding identification code into the study, this register will be kept on the Investigator File.

PUBBLICATION OF RESULTS

The results from this study can be published or shown at scientific conferences. According to usual practice, this multicentric study will be published as a whole, and not with the data obtained separately from each of the sites participants. It is expected that other articles are published about the exploratory aspects of this trial once the main data has been published. The final publication of the trial results will be written by the Coordinator Investigator.

All publications (papers, abstracts, presentations…) including data from the present trial will be submitted for review to all co-authors prior to submission.

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